Elevated hydrostatic pressure selectively alters TGF-β2, ET-1, and CTGF gene expression in human trabecular meshwork cells

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Introduction

Glaucoma is a leading cause of blindness worldwide, projected to affect nearly 80 million people by the year 2020.[1] In the US, it is estimated that nearly 2 million individuals age 45 years and older have primary open angle glaucoma (POAG), the most prevalent form of the disease.

The pathophysiology of POAG remains unclear. Elevated intraocular pressure (IOP), however, is considered a poorly understood hallmark of POAG. Within healthy eyes, IOP is maintained by a balance of aqueous humor (AH) production and outflow. Increased AH outflow resistance is considered a major contributor to IOP elevation.

Previous studies have shown that TGF-β2, a pro-fibrotic cytokine, enhances outflow resistance and increases IOP, in part, by inducing endothelin-1 (ET-1) and connective tissue growth factor (CTGF) expression and release within the TM.[2-5]

The effect of elevated pressure itself, however, on TM cell responses is not well understood. *Here, we show that human* TM *cells acutely exposed to elevated hydrostatic pressure exhibit marked increases in endogenous CTGF, ET-1, and TGF-*β2 *expression and release.*

Methods

General Design: Primary (hTM) or SV-40 transformed human TM (GTM3) cells were cultured in DMEM containing GlutaMAX-I supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin under an atmosphere of 5% CO_2 / 95% air at 37°C. Confluent cultures were serum-starved x 24h, media replaced with fresh DMEM, and immediately sealed in a chamber under an atmosphere of 5% CO_2 / 95% air at 37°C at either ambient pressure (1 atmosphere/760 mm Hg), or at elevated pressures of +30 mmHg above ambient for up to 48h.

F-Actin Staining: Cultured TM cells were fixed in freshly prepared phosphate-buffered (10 mM, pH 7.4) 4% paraformaldehyde (PFA) x 10 min at 23°C. Fixed cells were stained (x 20 min) with 165 nM phalloidin 488, counterstained with 4',6-diamidino-2-phenyindole (DAPI). Qualitative changes in F-actin staining was captured using a Leica TCS SPE confocal microscope and imaging suite.

Real-Time RT-PCR: Total RNA was extracted from treated cells using TRIzol reagent and 1 µg was reverse-transcribed. cDNA sequences were amplified by real-time PCR with a BioRad CFX Connect PCR detection system using human specific primer sets. In each case, the specificity of the real-time reaction product was confirmed by melt curve analysis. Relative fold-changes in mRNA content were normalized to GAPDH. In some experiments, a predesigned microarray containing 16 unique "POAG" primer sets (BioRad) was evaluated according to the manufacturer's instructions.

ELISA: ET-1, active or total TGF-β2 in cell culture supernatants from cells treated as described above were quantified using commercially available human-specific ELISA kits.

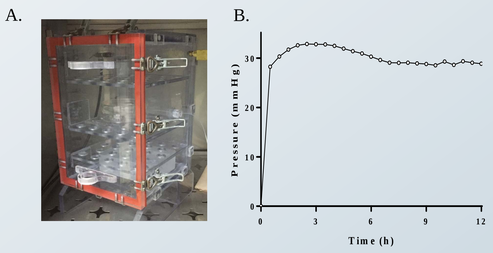
Immunoblot: Cells treated as described above were lysed in 2x Laemmli's sample buffer. Culture media was collected and concentrated by vacuum centrifugation. Proteins (30 µg per lane) in cell lysates or concentrated media were resolved by PAGE, electro-transferred onto nitrocellulose membranes, and immunostained using a 1:1000 dilution of rabbit anti-human caspase 3, 1:500 dilution of goat anti-human CTGF, or 1:10,000 dilution of rabbit anti-human GAPDH primary antibody. Washed membranes were incubated x1h at 23°C in a 1:10,000 dilution of peroxidase-conjugated goat anti-rabbit or donkey anti-goat IgG secondary antibody, respectively. Immunostained proteins were visualized by ECL.

LDH assay: Lactate dehydrogenase (LDH) released into the culture media from cells treated as described above was quantified colorimetrically.[6] Aliquots of culture media were incubated with iodonitrotetrazolium chloride x 1h at 23°C and formazan precipitate dissolved in DMSO and absorbance read at 490 nm.

MTT assay: Mitochondrial function was determined colorimetrically using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Adherent cells were incubated in the presence of 1 mM MTT x 2h at 37°C followed by addition of DMSO. Absorbance was read at 570 nm.

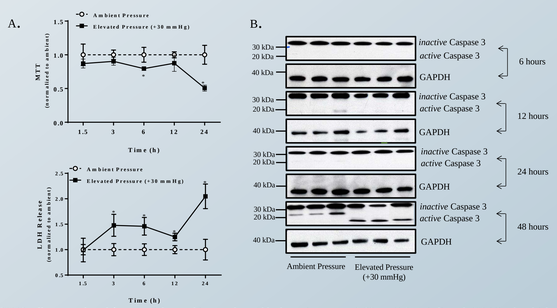
Results

Figure 1: Hydrostatic Pressure Chamber



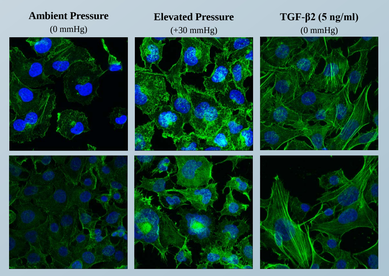
(A) Custom built chamber used to culture human TM cells at elevated hydrostatic pressures. **(B)** Representative pressure trace of GTM3 cells cultured at elevated (+30 mmHg) pressure for 12 hours

Figure 2: Effect of elevated hydrostatic pressure on TM cell viability



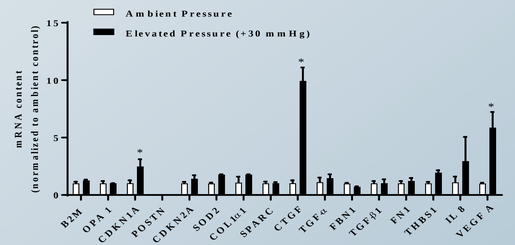
GTM3 cells were cultured for up to 24h at ambient or elevated hydrostatic pressure (+30 mmHg), as indicated. **(A)** Mitochondrial function or membrane integrity were assessed by MTT formation and LDH release, respectively. Relative values were normalized to ambient pressure controls. **(B)** Western immunoblot of total and cleaved caspase 3. GAPDH was used as loading control. Data shown are the means ± SEM (N = 8). * p <0.05, two-way ANOVA with a Bonferroni post hoc.

Figure 3: Elevated hydrostatic pressure increases F-actin



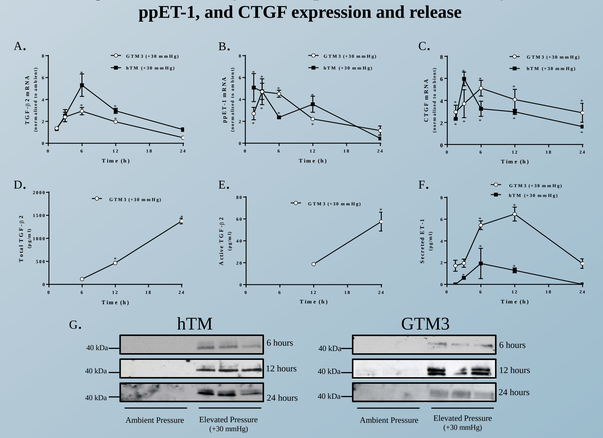
Representative duplicate confocal images of GTM3 cells cultured at ambient or elevated hydrostatic pressure (+30 mmHg), as indicated, and stained for filamentous (F) actin (phalloidin, green) and counterstained with DAPI (blue). GTM3 cells treated with TGF-β2 (5 ng/ml, 24h) and stained for F-actin are shown for comparison.

Figure 4: Elevated hydrostatic pressure selectively enhances expression of "POAG" associated genes



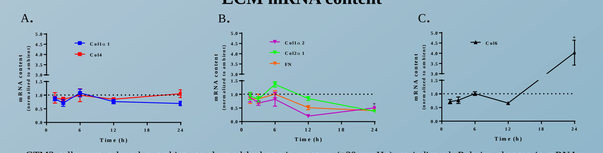
GTM3 cells were cultured at ambient or elevated hydrostatic pressure (+30 mmHg, x 6h), as indicated. Relative changes in mRNA content were analyzed utilizing a predesigned "POAG" nano-array. Data shown are the mean fold-changes ± SEM (N=4) from two independent experiments. * p<0.05, Student's *t-test*.

Figure 5: Elevated hydrostatic pressure enhances TGF-β2, ppET-1, and CTGF expression and release



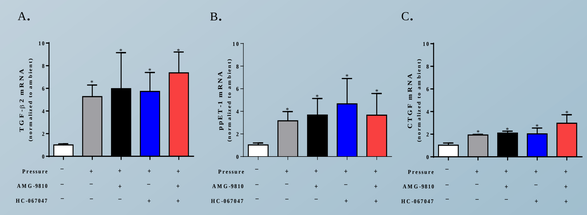
Human primary (hTM) or transformed (GTM3) TM cells were cultured at ambient or elevated hydrostatic pressure (+30 mmHg), as indicated. **(A-C)** Relative changes in mRNA content were quantified by qPCR. **(D-F)** Proteins secreted into the culture media were quantified by ELISA. **(G)** Relative content of secreted CTGF was measured by immunoblot. Data shown are the mean fold changes ± SEM (N=6). *, p<0.05 relative to ambient pressure control; two-way ANOVA with Bonferroni post hoc analysis.

Figure 6: Elevated hydrostatic pressure selectively alters ECM mRNA content



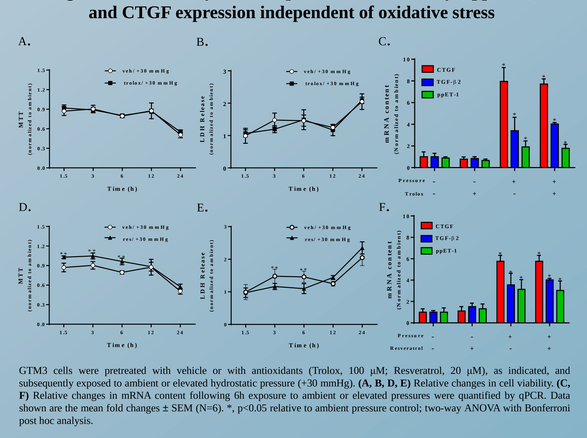
GTM3 cells were cultured at ambient or elevated hydrostatic pressure (+30 mmHg), as indicated. Relative changes in mRNA content were analyzed by qPCR. Data shown are the mean fold-changes ± SEM (N=6). *, p<0.05 relative to ambient pressure control; two-way ANOVA with Bonferroni post hoc analysis.

Figure 7: Elevated hydrostatic pressure alters TGF-β2, ppET-1, and CTGF expression independent of TRPV1 or TRPV4



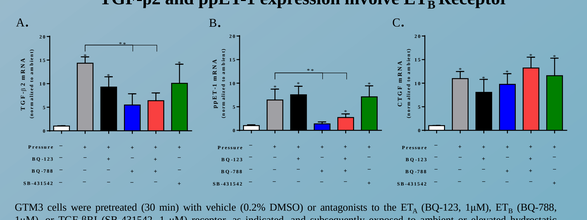
GTM3 cells were pretreated (30 min) with vehicle (0.1% DMSO) or with antagonists to TRPV1 (AMG-9810, 1µM), TRPV4 (HC-067047, 1µM), or the combination, as indicated, and subsequently cultured at ambient or elevated hydrostatic pressures (+30 mmHg, x 6h). Relative changes in mRNA content were quantified by qPCR. Data shown are the mean fold changes ± SEM (N=6). *, p<0.05 relative to ambient pressure control; two-way ANOVA with Bonferroni post hoc analysis.

Figure 8: Elevated hydrostatic pressure alters TGF-β2, ppET-1, and CTGF expression independent of oxidative stress



GTM3 cells were pretreated with vehicle or with antioxidants (Trolox, 100 µM; Resveratrol, 20 µM), as indicated, and subsequently exposed to ambient or elevated hydrostatic pressure (+30 mmHg). **(A, B, D, E)** Relative changes in cell viability. **(C, F)** Relative changes in mRNA content following 6h exposure to ambient or elevated pressures were quantified by qPCR. Data shown are the mean fold changes ± SEM (N=6). *, p<0.05 relative to ambient pressure control; two-way ANOVA with Bonferroni post hoc analysis.

Figure 9: Elevated hydrostatic pressure mediated increases in TGF-β2 and ppET-1 expression involve ET_B Receptor



GTM3 cells were pretreated (30 min) with vehicle (0.2% DMSO) or antagonists to the ET_A (BQ-123, 1µM), ET_B (BQ-788, 1µM), or TGF-βRI (SB-431542, 1 µM) receptor, as indicated, and subsequently exposed to ambient or elevated hydrostatic pressure (+30 mmHg, x6h). Relative changes in mRNA content were quantified by qPCR. Data shown are representative results of two independent experiments expressed as mean fold changes ± SEM (N=3). *, p<0.05 relative to ambient pressure control; two-way ANOVA with Bonferroni post hoc analysis.

References

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Summary/Conclusion

Cultured human TM cells acutely challenged with elevated hydrostatic pressure exhibit increases in F-actin as well as selective increases in TGF-β2, ET-1, CTGF, and Col type VI expression. Elevated hydrostatic pressure mediated changes in gene expression occur by a mechanism that involves activation of the ET_B receptor but is independent of TRPV 1, TRPV 4 or oxidative stress. Collectively, these results suggest that ***Pressure-dependent changes in TM cell gene expression represent a feed-forward mechanism that exacerbates TGF-β2 associated increases in TM cell contractility and altered ECM remodeling in affected POAG patients.***

Acknowledgments

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